THE LONGEVITY OF BACILLUS TYPHOSUS IN NATURAL WATERS AND IN SEWAGE.

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INTRODUCTION.

IN a paper published in this *Journal* in 1904,[1] Jordan, Russell, and Zeit detailed an extensive series of experiments on the longevity of the typhoid bacillus in water, in which simultaneous, though independent, tests were made on this organism as exposed to the waters of Lake Michigan, the Chicago River, the Chicago Drainage Canal, and the Illinois River. The methods used in this study and the results obtained were so different from those which have previously been reported that it seems desirable to test this question further, employing waters of different origin.

The attempt was made in all of this work to approximate, as closely as possible, the conditions that exist in nature, and, for this reason, a marked change in technique was instituted. Heretofore, it has been customary for experiments on the longevity of bacteria to be made in glass containers, filled with sterile or raw waters. The conclusions based on work under these conditions have been shown to be erroneous, and in the work previously referred to, the method was adopted of exposing the typhoid organism in permeable sacs (celloidin and vegetable parchment), filled with the type of water in which the sacs were suspended. If, then, any variation occurred in the composition of the stream in which the sacs were exposed, the influence of such variation, if of any effect, should be felt on the imprisoned cultures within the sac.

The results obtained in the experiments conducted on the Chicago Drainage Canal and other waters showed a marked variation in the vitality of B. typhosus. In the relatively pure waters of Lake Michigan, this organism could be recovered readily from the infected sacs, for a period of at least a week, while in the highly polluted waters of

[1] *Jour. Infect. Dis.*, 1904, 1, p. 641.

the Chicago River and the Drainage Canal, the longevity of the same strain, exposed in a similar way, was reduced to two days. These results were obtained with uniform regularity by all three observers, working independently, but employing the same general methods. The conclusions then drawn were of a tentative character, and it was deemed advisable to carry on further work. The studies here reported follow, in general, similar lines, using waters of different origin, under as widely diverse conditions as possible.

Special attention has also been given to the development of technical methods other than those previously used, so as to broaden, as far as possible, the basis upon which conclusions were to be made. Inasmuch as most of the technical methods used in the experiments here described are practically the same as those previously reported in the foregoing paper, it will not be necessary to repeat them in this connection. Only those modifications that further experience has demonstrated to be valuable, and the new methods that have been developed are here referred to.

These experiments have been made in the Wisconsin State Hygienic Laboratory at the University of Wisconsin. In part of the preliminary work much assistance was received from Mr. G. J. Marquette, then assistant in this laboratory. The waters used in these tests were from Lake Mendota, a spring-fed inland lake, of about 25 square miles extent, the waters of which may be regarded as fairly typical of those of a surface character. The sewage-infected waters were produced by adding to the lake water a given quantity of fresh liquid and solid human excreta.

METHOD OF EXPOSING THE TYPHOID BACILLUS.

In these experiments the exposure of the typhoid bacilli was made in the laboratory, rather than in the lake itself, the water, however, being piped for only a short distance. To place the infected sacs under conditions convenient for sampling and where they would not be subjected to the action of the weather, which was more or less troublesome in the Chicago series, the sacs containing the waters infected with the typhoid bacilli were placed in large tubulated glass receptacles, holding from two to three gallons, through which there was allowed to flow continuously a stream of water or sewage.

Reference to accompanying figure will indicate the arrangement of this device.

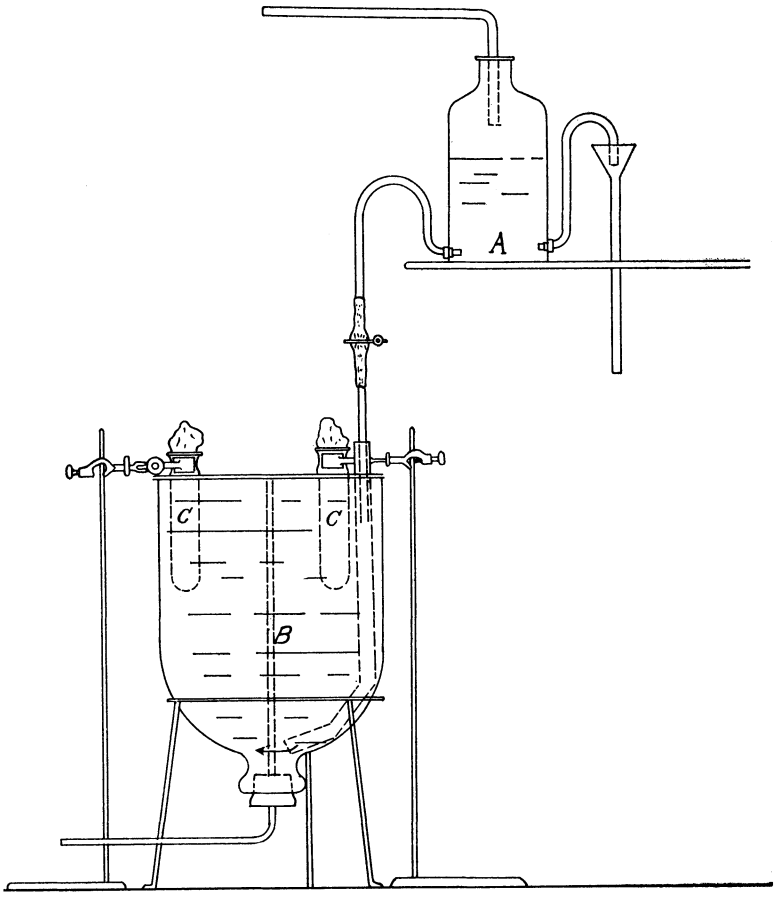

FIG. 1.

The sacs were immersed in the flowing stream so that the level of liquid in same was slightly below that of the water outside, thus maintaining a slight pressure toward the inside.

When exposed to the action of the lake water, the connection was made directly with the tap supplying this kind of water. To expose the sacs to the influence of sewage-infected waters, the device as shown in the cut was used. The sewage mixture was made up in a large reservoir placed in the attic, and from this was discharged through a pipe into a safety bottle, *A*, which regulated the flow into the reservoir below, *B*. The sacs, *C*, were held in position by clamping a rubber-faced clamp to a glass neck, which was sealed on to each kind of sac. The rate of flow was about six gallons per hour in the case of sewage, and a considerably higher rate for the lake water.

In the previous experiments, permeable sacs of celloidin and vegetable parchment were employed. In the work here recorded, another method has been devised, that of agar membranes. Some modifications of the previous methods have also been adopted.

1. *Celloidin sacs.*—The celloidin sacs employed have all been made by the extremely simple method of Frost,[1] in which the celloidin solution is poured on the inside of the test tube and the film, after it has been air-dried for the proper time, shrunk from the glass wall by means of water. By means of this method, sacs of practically any size can be made in a few minutes. These celloidin tubes were usually made to hold about 50 c.c. of water. They were held in position by inserting a glass neck of approximately the same bore as the sac, tying this on tightly with a soft-fibered thread, and coating the same with a layer of celloidin, allowing it to air dry. The sacs are filled with, as well as immersed in, water during the process of sterilization, which is done in an Arnold.

2. *Parchment sacs.*—In the former work sections of parchment tubing were used, such as is employed in dialysis work, but it is frequently difficult to secure tubing that is perfectly sound and free from minute holes. In this work, we have employed the parchment diffusion shells made by Schleicher and Schüll. These hold approximately 50 c.c. and are in the form of a tube closed at one end. Into the open, free end, a glass neck is fastened by means of sealing wax. The shells themselves are sterilized in streaming steam for an hour and a half, then allowed to dry under cover from the air. The glass necks can be sterilized chemically. The two parts can be quickly assembled in a sterile condition.

3. *Agar membrane sacs.*—The first introduction of agar for dialyzing purposes in bacteriological studies was made by Frost,[2] who used rectangular blocks about one-half inch square and an inch and a half long. These were made of plain agar and were inoculated by means of a stab in the center. The upper part of the stab was sealed off by dropping on melted agar, or smearing the upper surface of the block with a hot iron. Our first studies with agar were made with blocks of a similar character, but it was found that the

[1] *Amer. Pub. Health Assoc. Rep.* 1903, 28, p. 36.

[2] *Jour. Infect. Dis.*, 1904, 1, p. 599.

layer of agar was too thick to permit rapid and complete dialysis. In some cases the typhoid organism, inoculated as a stab culture, died in a short time, while in other cases it persisted for a long period (several weeks).

The attempt was then made to use an agar film instead of the thicker block. It is necessary to have some mechanical framework to support the thin, delicate film of agar, and for this purpose cellulose diffusion shells, such as are used in chemical manipulations, have been found very serviceable. These are of the same size as the parchment shells previously referred to (38 by 85 mm.). After they have been sterilized in steam and allowed to dry, sterile glass necks of the same bore are inserted into the shells and fastened by means of sealing wax. The shell is then ready to receive its agar coating. Care is taken in the preparation of the agar to remove from it as much organic matter as possible. This is done by soaking the thread agar in distilled water for some hours, changing the water several times. A 2 per cent solution is then made. Tests made as to the nutritive properties of this agar showed that it would not support bacterial growth. Occasionally molds will make a sparse growth on the medium when left exposed to the air for some time. In coating the shells with the agar film, the material should be used in as hot a condition as it can be handled, so as to impregnate thoroughly the pores of the cellulose filter. It is advisable to pour some of the hot agar on the inside of the sac, rotating the sac quite rapidly, as in a roll culture, so as to distribute the material uniformly. The porous cellulose wall absorbs the liquid rapidly. After a little experience, one learns the requisite quantity of medium to employ in order to give a uniform and sufficient coating, and not have an excess. It is advisable to have the coating made at a single immersion, as the film is more homogeneous than where several applications are made.

When the inside coating has been properly applied, the sac is then dipped into the liquid agar and rotated so as to coat the outside of the sac also with a uniform layer. If the inside of the sac has not been coated in such a way as to exclude the air entangled in the cellulose meshwork, air vesicles will develop in the outer agar coat after a time and the integrity of the sac will be destroyed. Care

should be taken in this coating process to prevent infection of the sac, as it is of course impossible to sterilize the sacs after they are once made.

These agar films are not as durable as celloidin or parchment, but their integrity will be maintained unimpaired for about two weeks, or even longer, depending upon the nature of the liquid in which they are immersed. In water they retain their germ-tight properties longer than they do in sewage. In liquids very rich in bacteria, such as sewage, cytolytic enzymes are undoubtedly produced by certain types of organisms, thus softening the cellulose matrix and causing the sac to disintegrate.

While these agar membranes possess no point of superiority over the parchment or celloidin membranes formerly used, it is of importance to broaden the technical methods just as much as possible, and thereby determine if there are any essential variations in the results obtained which are due to the nature of the methods employed.

Permeability of different types of sacs used.—We have introduced into this study the use of a new type of permeable membrane, the agar film, and as no records have been reported on the question of relative permeability, so far as we know, it has been deemed advisable to incorporate here some of the results obtained in the study of these different types of sacs. The sacs used in this experiment are intended to hold in captivity the typhoid organism, and still at the same time subject this germ to the influence of diffusible substances that may be in the enveloping medium without.

In testing the permeability of these membranes, we have used simple,well-known substances that could be determined quantitatively, and have made no attempt to study the diffusibility of such materials as might possess an inhibitory effect on the imprisoned typhoid organism.

a) Tests with chlorides.—Experiments were first made with sodium chloride. Sacs were filled with distilled water and immersed in tap water, to which enough salt had been added to make the chlorine test 93 parts per million. Tests for chlorine were made on the contents of the immersed sacs at intervals of ½, 1, 6, 12, and 24 hours. The results of these determinations are expressed in Table 1.

From the results herein shown it appears that a condition of nearly perfect equilibrium was established in all three sacs within a comparatively short time. The diffusion of the chlorides was somewhat more rapid in the agar and celloidin sacs than in the parchment, but within 24 hours' time, where no artificial currents were

TABLE 1.

CHLORINE (PARTS PER MILLION) FOUND IN PERMEABLE SACS AFTER VARYING PERIOD OF IMMERSION IN SALT SOLUTION (93 PARTS PER 1,000,000).

	Agar	Parchment	Celloidin
½ hour	0	0	0
1 "	2.5	4	6
6 hours	63	50	76
12 "	80	67	84
24 "	84	76	86

used either within or without the sac to facilitate diffusion, from 81 to 92 per cent of the chlorine passed through the membranes of the sacs. In Fig. 2 are shown the data presented in the above table, expressed on a percentage basis of the total strength of the solution.

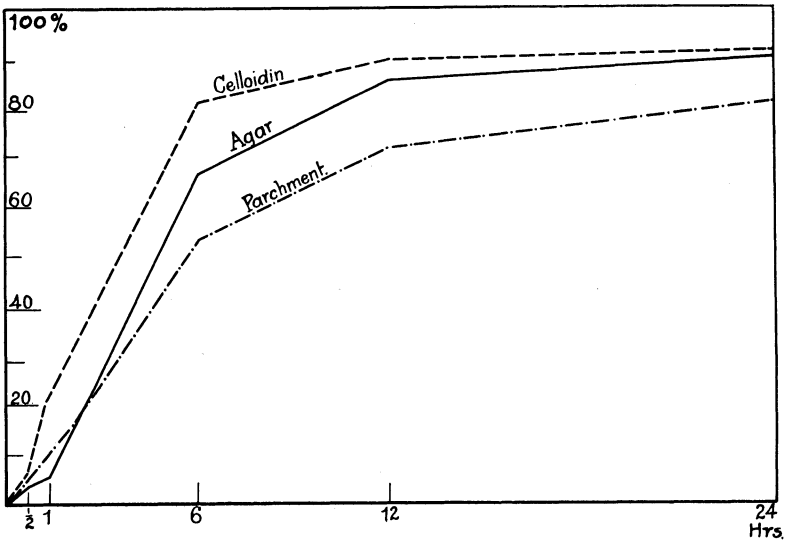

FIG. 2.—Relative permeability of different kinds of sacs to NaCl solutions

b) *Tests with sugars.*—In addition to the chlorides, tests were also made in the same way with sugar. Sacs filled with tap water were immersed in tap water containing 1.75 grms. of saccharose per 100 c.c. Quantitative determinations were made at ½, 1, 6, 12, and 24 hour intervals with the following results:

TABLE 2.

SUGAR (IN GRAMS PER 100 C.C.) FOUND IN PERMEABLE SACS AFTER VARYING PERIOD OF IMMERSION IN SOLUTION CONTAINING 1.75 GRAMS PER 100 C.C. WATER.

	Agar	Parchment	Celloidin
½ hour	0.03675	0.02625	0.20475
1 "	0.0630	0.0525	0.3850
6 hours	0.39475	0.22225	1.26525
12 "	0.6650	0.4270	1.4805
24 "	1.1375	1.0115	1.5925

These data, which are shown in graphical form in Fig. 3, seem to indicate that the celloidin type is much more permeable to sugar solutions than either agar or parchment. The rate of diffusion was not quite so rapid in this series as in the salt series, except in the case of the celloidin type.

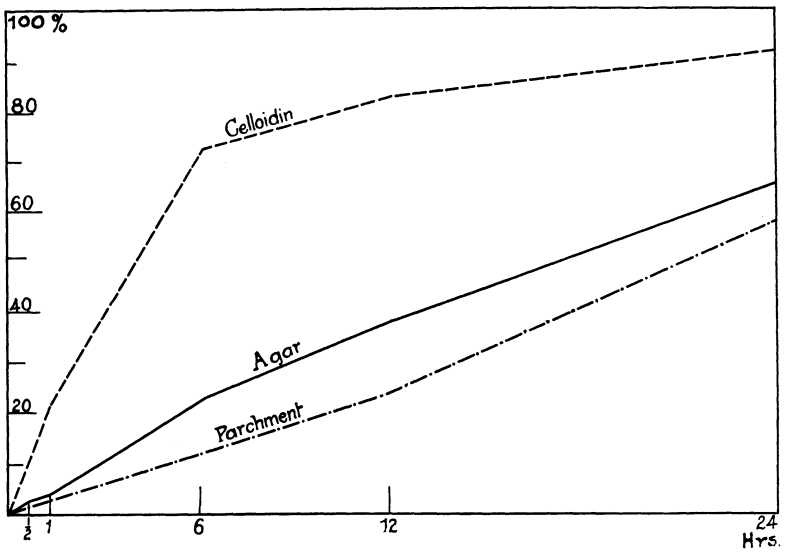

FIG 3.—Relative permeability of different kinds of sacs to sugar solutions.

c) Tests with peptone.—Further tests were made with peptone solutions in order to test the permeability of the sacs to diffusible proteids. Sacs were filled with tap water and immersed in the same type of water, containing 1 per cent peptone. In these tests, a qualitative determination only was made. The contents of the sacs were tested for peptone by means of the biuret reaction at intervals of five minutes, until a positive reaction was obtained. The celloidin sac gave a positive reaction in 25 minutes, while the agar and the parchment required 35 minutes. The intensity of the reaction increased markedly in all cases after an hour's exposure.

It would appear from these tests, where various chemical substances of a widely different nature were employed, that the permeability of these different membranes was pronounced. On the whole, the results indicate that celloidin is the best membrane to employ, but this type is well supplemented by the addition of agar and parchment. Further corroborative evidence on the matter of permeability may also be presented in results that were noted in the actual prosecution of the work.

Growth of bacteria in sacs immersed in nutrient solutions.—If sacs of this character are sufficiently permeable to permit food substances in solution to pass the limiting membrane, it ought to be

possible to obtain growth of typhoid and water bacteria in sacs filled with water and immersed in liquids containing available organic matter.

To test this point more specifically, a special experiment was instituted. Six celloidin sacs were prepared and filled as follows: Two with sterilized tap water and the remaining four with raw tap water. The two sacs filled with sterilized water and two filled with raw tap water were inoculated with the same strength of typhoid suspension; the two remaining raw-water sacs were not infected with typhoid. These sacs were divided into two sets of three each. One set was immersed in running raw water, the other placed in sterile water containing 0.2 per cent peptone solution.

The results obtained in this experiment showed a most marked difference in the two sets of sacs, the peptone and the water series. In all three sacs of the peptone series a very marked growth was observed, both in the case of water bacteria originally present in the raw water, and the inoculated typhoid. The germ content of the control sac filled with raw water rose from a 32 colony count on plain agar on the first day, to 12,000,000 in the course of three days, and in 18 days had reached 40,000,000 bacteria per c.c. The sac filled with sterile water and infected with typhoid (16,000 per c.c.) underwent even a more pronounced growth than this. On the fourth day it contained 137,000,000 typhoid bacilli per c.c. From this high point the germ content gradually declined, but in 18 days there were still over 6,000,000 colonies per c.c., and the purity of the culture demonstrated the integrity of the sac. The course of changes followed by the sac containing the mixed flora (water bacteria + B. typhosus) underwent the same general change. On the 18th day 45,000,000 organisms per c.c. were demonstrable, and B. typhosus had been recovered in abundance on each intermediate day the test had been applied.

The course of changes noted in the water sacs was entirely different. The content of these sacs and the dosage was identical with the series immersed in the peptone solution. The only difference in this case was that the sacs were immersed in flowing tap water. The sac filled with raw water showed no growth on Drigalski-Conradi medium, and less than 50 bacteria per c.c. on plain agar.

Cultures were made for a period of six days, but no essential alteration in germ content was observed. The sac filled with sterile water and inoculated with B. typhosus (25,000 per c.c.) showed no increase. On the sixth day of the experiment it contained 17,000 organisms per c.c., apparently a pure culture (17 colonies picked all proved to be positive typhoids).

Accidentally the membrane was perforated on the seventh day, but for the period of observation reported no marked change had occurred in the germ content of the sac. The sac filled with raw water and infected with 30,000 B. typhosus fell to 3,000 on the third day. On the fifth day B. typhosus was recovered, but on the sixth day none could be found in culture plates containing about 100 colonies per c.c.

The results of this series are wholly consistent, and show that both water bacteria and B. typhosus are capable of multiplying extensively in raw waters, as well as sterile, where such waters are exposed in permeable sacs in liquids containing available food material in solution.

This experiment conclusively demonstrates the permeability of the sacs to such solutions, and would seem to show beyond all reasonable doubt that if bacteria die rapidly when imprisoned in such permeable cages, they do not succumb because of the inability of food substances in outside enveloping liquid to pass the limiting membranes of these sacs.

In addition to this carefully controlled experiment with peptone solutions, observations were made in the course of the experiments later detailed, which also throw light on this point. In series VIII a sac filled with raw water, but inoculated with typhoid, was immersed in a bath of flowing sewage. In Table 16 is shown the bacterial content of this sac on various days. A phenomenal development of the water bacteria occurred in this case, as in the peptone solution, showing that sewage also contains sufficient good material, which was able to permeate the celloidin membrane to give the water bacteria in raw water a favorable environment for rapid growth.

These results, taken in connection with the specific tests made as to permeability, would seem to indicate that the methods employed

permitted diffusion to occur with sufficient rapidity so that the conditions approximated those that prevail in a flowing stream.

Germ-proof qualities of sacs.—In using sacs of a permeable character, it is of the utmost importance that they should be relatively germ tight. The celloidin sac has been tested for so long a period of time that there is no longer any question as to the tightness of sacs of this type, but concerning the use of parchment and agar, this question may well be raised. In our earlier experiments, the attempt was made to rely on parchment tubing, such as is used in dialysis work, but we have been much troubled to get satisfactory tubing of this character. The process of making the cellulose fiber into vegetable parchment seems to destroy the pliability of the material, so that it cracks more readily upon bending. In this way minute breaks or punctures are often to be noted. Since the adoption of the parchment diffusion shells, no trouble of this character has occurred.

The agar sacs are, of course, relatively fragile so far as the film is concerned, and the filter-paper matrix on which the agar is spread is not as permanent as parchment. When immersed in liquids rich in bacterial life, such as sewage, the cytolytic enzymes cause the disintegration of the cellulose fibers, this occurring more rapidly in the filter paper than with the parchment sac. In purer types of waters this rotting does not occur so readily. We have rarely had any trouble with sacs of this character, if the experiment did not exceed two weeks' time.

When the sacs are allowed to remain in dilute sewage, or even in water after a considerable lapse of time (10 days or more), a somewhat slimy growth is formed, as is also the case on the inner face of the glass receptacle. This is easily removed by brushing the sacs occasionally with a camel's hair brush.

Experiments were made with the special object of testing the integrity of these types of sacs. Sterile sacs were filled with sterilized water and immersed in either water or sewage. The result of such tests, even where continued for a week or more, showed no passage of bacteria through the sac membrane.[1]

[1] Johnson (*Eng. Rec.*, Sept. 23, 1905. also *Jour. New Eng. Water Works Assoc.*,, 1905, 19, p. 508) has questioned the integrity of sacs of this character, but the experimental data given by this writer are too meager to permit of any weight being attached to his conclusions.

In some of the actual tests performed in these studies (see Series VII and VIII), it was possible to throw light on the matter of tightness of the sacs. Sacs filled with lake water and inoculated with typhoid were immersed in sewage. Drigalski-Conradi plates were made at daily intervals. If any leakage had occurred, it would have immediately manifested itself by the appearance of acid colonies on this medium, as, of course, the colon type was abundant in the dilute sewage outside of the sac. In most of the sacs no evidence of any leakage occurred. In one or two of them slight evidence of leakage was discovered in the course of 13 to 14 days. We are, however, of the opinion that any one of these three methods may be relied upon to maintain readily a germ-tight, and yet permeable, membrane, if any degree of care is used in selection and manipulation of the sacs.

CULTURES EMPLOYED.

In order that this work might be directly compared with that of the previous year, one of the same typhoid strains that was employed in the Chicago Drainage Canal work has been used throughout all of these experiments. This culture, strain "Y," was isolated October 1, 1903, under Professor Jordan's direction, from the urine of a typhoid patient. The case was typical in its symptoms, and the blood of the patient gave a positive agglutination test on the tenth day.

METHOD OF RECOVERY OF TYPHOID BACILLUS FROM SACS.

Where the typhoid organism is in direct contact with water or sewage forms, it is advisable to employ some of the special methods that have been devised for the differential cultivation of this organism. For this purpose we have employed throughout this work, the Drigalski-Conradi medium,[1] modified somewhat by the omission of nutrose. By filtering the mixture before adding the litmus solution, the flocculent precipitate is much reduced.

The use of a culture medium like the D.-C. medium is of great value in inhibiting bacterial growth which would otherwise obscure the typhoid organism. While the addition of the crystal violet does not completely inhibit water bacteria, it reduces materially the germ content, as is shown from the following data, where cultures were made on plain nutrient agar and on the D.-C. medium.

[1] *Ztschr. f. Hyg.*, 1902, 39, p. 282.

TABLE 3.

RELATIVE GERM CONTENT OF WATER SACS ON AGAR AND ON DRIGALSKI-CONRADI MEDIUM.

Date	Amount Used	Medium Employed	Sac 1	Sac 2	Sac 3	Sac 4
1904						
December 15.....	1 c.c.	Plain agar	6,500	3,900	730	1,620
	1	D.-C. medium	750	410	150	350
December 16.....	1	Plain agar	6,500	1,050	1,530	1,150
	1	D.-C. medium	560	300	137	175
December 17.....	1	Plain agar	5,900	4,100	1,450	6,500
	1	D.-C. medium	1,150	4,300	490	150

RAPID IDENTIFICATION OF TYPHOID ORGANISM.

In determining whether any organism isolated from the plate cultures is true typhoid or not, it has generally been customary to test the presumptive organisms by first passing them through dextrose agar shake cultures, or stabs. From this, if no gas was produced, transfers were made into litmus milk and gelatin, and tests for indol were made. To differentiate it from B. alkaligenes, which develops no acid in litmus dextrose broth, cultures were made in this medium. If the organism in question stood all these culture tests satisfactorily for typhoid, it was then finally tested for agglutination with a highly potent typhoid-immune serum. Such a procedure as this involves a large number of transfers. In the course of these studies, a modification of the above method was made, which is as follows:

The Drigalski-Conradi plate cultures were carefully studied to pick out the presumptive typhoid colonies. This was not always easy to do, as there are many organisms occurring in sewage, or even in water, that are capable of development in this crystal violet medium, and which retain the blue color. Many of these can, of course, be easily rejected, as they are too luxuriant in their growth, being thick and opaque. But, not infrequently, types of colonies of a thin, semi-transparent blue cast have appeared, that more or less closely resembled the true typhoid. In picking out the presumptive typhoids, it is advisable to have on hand, for purposes of comparison, several culture plates made from a pure typhoid strain.

A more rapid method of identification was devised as follows: The presumptive typhoid-like colonies were fished and subcultured directly into litmus *dextrose* agar, by making a combination streak and stab culture. In this medium the typhoid, of course, formed acid, but no gas, and was thus easily differentiated from Petruschky's B. fecalis alkaligenes, which remained blue, not only in lactose but in glucose litmus media. Organisms of the colon type would naturally acidify dextrose agar, but these are excluded on the D.-C. culture plates. If, perchance, colon types should be transferred, their presence would be manifest by the copious gas production.

Following the litmus glucose test, all non-gas-producing acid forms were then subjected directly to the macroscopic agglutination test with typhoid immune sera. If the isolated cultures stood these two tests, they were considered positive typhoids. Upon the completion of these tests, a number of cultures from each series were selected at random and subcultured on all the usual media, as gelatin, milk, and

glucose-free broth for indol, so as to check still further the culture characteristics of the supposed typhoid cultures.

Our experience with this short method of identification leads us to recommend its use over the longer method. We have found no organism in normal waters that is liable to be confused with the typhoid, where reliance is placed on these tests. In sewage, however, there is a blue type of organism that appears in 24 to 48 hours on Drigalski-Conradi plates, and which, therefore, might be transferred to the litmus dextrose cultures, on which it forms acid. This type has invariably failed to be agglutinated with typhoid-immune sera. Later, if one studies the original plate cultures, after a more prolonged period of incubation, he finds that these colonies are faintly acid. Evidently they are able to produce acid very slowly on lactose media.

OUTLINE OF EXPERIMENTS MADE.

In order to present a general summary of the work done in this series of studies, a synoptical table is presented below, in which the varying conditions, as to manner of exposure, dosage, temperature, etc., are shown.

TABLE 4.

SYNOPSIS OF DIFFERENT SERIES OF EXPERIMENTS MADE.

	No. of Series	No. Sacs Used	Kind of Sacs	Approximate Typhoid Dosage per c.c.	Nature of Liquid in Sac	Nature of Liquid in Outside Container	Range in Temperature (° C.)
LAKE WATER	I	2	Agar	110,000–200,000	Lake water	Lake water	10–14
		2	Celloidin	110,000–200,000	" "	" "	10–14
	II	1	Agar	25,000	" "	" "	9–12
		1	Celloidin	85,000	" "	" "	9–12
	III	1	Celloidin	150,000	" "	" "	15–18
		2	Parchment	120,000–150,000	" "	" "	15–18
	IV	1	Celloidin	2,275,000	" "	" "	21–23
		1	Glass	1,706,000	" "	.....	21–23
SEWAGE	V	1	Parchment	1,500,000	Sewage	Sewage	21–29
		2	Celloidin	5,000,000	"	"	21–29
		1	Agar	10,000,000	"	"	21–29
	VI	1	Parchment	4,350,000	"	"	22–25
		1	Celloidin	5,800,000	"	"	22–25
SEWAGE IN WATER	VII	2	Celloidin	190,000–300,000	Lake water	Sewage	15–19
		2	Parchment	" "	" "	"	15–19
		2	Agar	" "	" "	"	15–19
	VIII	2	Celloidin	2,275,000	" "	"	22–25
		1	Parchment	1,706,000	" "	"	22–25
	IX	1	Celloidin	100,000	" "	"	22–25
		1	Parchment	200,000	" "	"	22–25
SEW'GE IN WATER	X	1	Parchment	4,350,000	Sewage	Lake water	21–23

PART I.

BACILLUS TYPHOSUS EXPOSED TO LAKE MENDOTA WATER.

Series I. Agar and celloidin sacs filled with lake water and immersed in lake water.— This series was started on December 5, 1904. Two agar sacs and two celloidin sacs were filled with raw lake water, and each sac inoculated with two large loopfuls of a 24 hour culture of the "Y" strain of the typhoid bacillus. These cultures were exposed continuously to the action of flowing lake water from the above date until December 22, making a period of 17 days in all.

The temperature of the water as it reached the exposed cultures was about 12° C. Quantitative plate cultures were made on the D.-C. medium throughout the whole experiment, in order to note the relative rate of decline of bacteria within the sacs. In the beginning three plates were made daily from each sac, using $\frac{1}{10}$, $\frac{1}{2}$, and 1 c.c. of the infected water, respectively. From this set of plates a very fair average could be determined, and the accuracy of the work thus checked by the quantitative findings with different dilutions. As the work progressed and the colony count per c.c. fell from the high initial number to lower limits, the quantity used in each seeding was materially increased. Under such conditions it is highly improbable that typhoid organisms were overlooked, where all typhoid-like colonies were removed and tested.

The quantitative findings of this series are presented in Table 5, from which it is apparent that the high initial content (which was, of course, largely composed of typhoid organisms at the beginning) underwent a rapid and continuous decline from the beginning to the seventh day. By this time the average number of organisms in the four different sacs had fallen from the initial seeding of 147,700 per c.c. to 2,170 per c.c. From this period to the end of the experiment, the number of organisms per c.c. remained very small, ranging at most from a thousand or so to a few hundred.

TABLE 5.

NUMBER BACTERIA PER C.C. IN TYPHOID-INFECTED SACS AFTER EXPOSURE TO FLOWING LAKE WATER.

Date of Test after Infection	Temperature	Sac 1 Agar	Sac 2 Agar	Sac 3 Celloidin	Sac 4 Celloidin
4 hours	12° C.	130,000	111,800	203,000	146,000
2d day	12	34,800	43,000	152,000	99,250
3d "	12	41,000	45,500	109,000	90,000
4th "	14	29,500	33,000	58,500	62,500
5th "	14	16,000	28,600	49,000	46,250
6th "	14	13,750	22,250	30,750	33,250
7th "	7	2,350	900	2,405	3,315
8th "	9	1,260	500	1,520	1,180
9th "	10	1,720	360	280	420
10th "	12	750	410	150	350
11th "	13	560	300	135	175
12th "	12	1,150	500	490	150

This comparative decline is also shown graphically in Fig. 4.

The marked diminution in numbers noted in the above table is unquestionably due to the rapid destruction of the typhoid bacilli inoculated into the raw water with which the sac was filled. The initial germ content of this water before inoculation with typhoid ranged from 500 to 1,000 bacteria per c.c., but unquestionably some increase in water bacteria would occur even in these permeable sacs. As the D.-C. medium was used throughout all these tests, these water bacteria would be largely prevented

from development, so that the apparent diminution doubtless registers the decline in typhoid content.

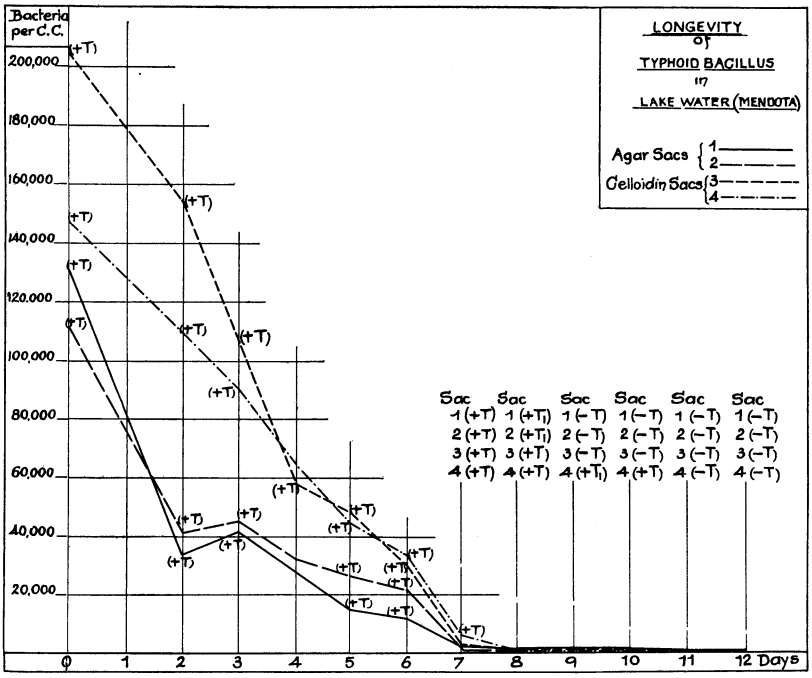

FIG. 4.

To check these quantitative results, extensive tests were also made to determine the longevity of the inoculated typhoid organisms by removing pure cultures from the Drigalski plates each day, and subculturing them.

The results of these qualitative tests are presented in Table 6, in which are given the number of organisms fished from the plates and also the number proven to be typhoid upon the basis of the agglutination test and subcultures. There was no difficulty in recognizing the typhoid organism in the plates during the earlier part of the series, as they were crowded with colonies of a similar character, and it was unnecessary to fish a large number. As the inoculated typhoid organism gradually died out, the colony appearance on the plates became more diverse, and the number of presumptive typhoid colonies was greatly diminished.

The results obtained in this series of tests are certainly very striking. A large proportion of the genuine typhoid colonies were found among those fished on the earlier days of the series. In the case of the agar sacs, as late as the seventh day, a majority of all fished colonies proved to be typhoid, while in the celloidin sac, this persistence was very marked, until a day later (eighth day). After

TABLE 6.

LONGEVITY OF B. TYPHOSUS IN AGAR AND CELLOIDIN SACS IN LAKE MENDOTA WATER

	SAC 1 (AGAR)		SAC 2 (AGAR)		SAC 3 (CELLOIDIN)		SAC 4 (CELLOIDIN)		TOTALS	
	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found
4 hours.....	3	3	4	4	2	2	4	4	13	13
2d day.....	8	7	9	9	9	8	9	5	35	29
3d "	10	9	9	8	8	8	5	4	32	29
4th "	11	4	10	4	11	9	6	6	38	23
5th "	5	1	10	8	8	8	9	8	32	25
6th "	9	5	10	8	10	9	8	7	37	29
7th "	10	4	8	5	7	7	12	8	37	24
8th "	3	1	4	1	4	3	6	6	17	11
9th "	2	..	2	..	2	..	2	1	8	1
10th "	1	..	1	..	5	..	5	1	12	1
11th "	2	..	..	..	..	..	..	..	2	..
12th "	6	..	..	..	3	..	7	..	16	..
13th "	1	..	2	..	..	..	..	..	3	..
17th "	..	..	..	..	6	..	..	..	6	..
	71	34	69	47	75	54	73	50	288	186

these dates a very pronounced diminution in typhoid colonies appears. Scattering colonies were found in the agar sacs on the eighth day, but none later, and in one of the celloidin sacs a similar condition was observed on the 9th and 10th days, but none could be found subsequently. The appearance of the culture plates in the earlier and later periods of this series showed a marked difference in colony aspect. In the first week the plates were studded with apparently typically typhoid colonies, which upon subculture proved to be genuine typhoid by the different culture tests and the agglutination reaction. After this period (8 or 10 days), the aspect of the colonies appearing on the culture medium was of an entirely different character, and only rarely did any forms appear that could be suspected of typhoid relationships. In no case, however, did any of these organisms prove to be typhoid.

Series II. Agar and celloidin sacs filled with lake water and immersed in lake water.—This series, which was run prior to that just described above, is not as satisfactory, in that the data acquired are not nearly as complete as they should be, but it is presented as furnishing evidence of the action of lake waters under winter conditions. The series consists of two sacs, one agar and one celloidin, both of which were filled with raw lake water and inoculated with a typhoid dosage of 25,000 and 85,000 typhoid bacilli per c.c., respectively. The sacs were then immersed, as before, in flowing lake water, having a range in temperature from 9° to 12° C. The data as to the presence of the inoculated typhoid bacilli are presented in Table 7.

TABLE 7.

LONGEVITY OF B. TYPHOSUS IN AGAR AND CELLOIDIN SACS IN LAKE MENDOTA WATER.

	TEMP. ° C.	SAC 5 (AGAR)		SAC 6 (CELLOIDIN)		TOTALS	
		No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found
3 days	14	7	4	10	2	17	6
5 "	12	6	0	2	0	8	0
8 "	12	11	4	9	1	20	5
12 "	14	4	0	5	0	9	0
		28	8	26	3	54	11

While the number of colonies removed in this case was not large, yet the same general result was obtained as before. It was readily observable on the plates made the third day after seeding that the number of typhoid colonies had undergone a marked reduction. While a daily study of this series was not made, yet the results, as far as they go, confirm in general the conclusion of the preceding series. A marked reduction in total colony count was observable after the third day. The last time typhoid was found was on the eighth day.

TABLE 8.

LONGEVITY OF B. TYPHOSUS IN PARCHMENT AND CELLOIDIN SACS IN LAKE MENDOTA WATER.

	SAC No. 7 (PARCH.)		SAC No. 8 (PARCH.)		SAC 9 (CELL.)		TOTALS—ALL SACS	
	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found
1 hour	4	4	4	3	4	3	12	10
1 day	6	0	6	2	4	4	16	6
2 days	5	1	8	1	4	2	17	4
3 "	8	2	8	0	5	5	21	7
4 "	5	2	8	1	7	0	20	3
6 "	6	2	10	4	7	1	23	7
8 "	10	1	6	0	9	0	25	1
10 "	4	0	3	0	3	0	10	0
11 "	4	0	3	0	3	0	10	0
13 "	4	0	3	0	1	0	8	0
15 "	1	0	..	..	1	0	2	0
17 "	1	0	2	0	..	..	3	0
	58	12	61	11	48	15	167	38

Series III. Parchment and celloidin sacs filled with lake water and immersed in lake water.—Another series in lake water was begun on May 2, 1905, in which the same general arrangement as before was followed. One celloidin and two parchment sacs were immersed in lake water, after being filled with lake water, which at this time contained 140 bacteria per c.c. The seeding of typhoid in this case ranged from 120,000 to 150,000 bacteria per c.c. Determinations made of the germ content of the lake water during the progress of the experiment, which lasted from May 2 to May 17,

showed at all times less than 400 bacteria per c.c. The temperature under these summer conditions ranged from 15° to 18° C., and was materially higher than in the two preceding series. The results of this test are shown in Table 8.

Here again there is practical unanimity as to the results obtained in the parchment and celloidin sacs. Typhoid colonies were detected in all three sacs on the sixth day and in one sac as late as the eighth day, although only one colony was found. Cultures were continued for a period of 17 days, but no typhoid colonies were recovered after the period mentioned. In this series the typhoid type could be differentiated on the culture plates with a greater degree of accuracy than in the preceding Series I. An attempt was made to estimate, only approximately, of course, the number of typhoid colonies that developed on the various plates. These data cannot be relied upon implicitly, because one cannot be absolutely sure as to whether a colony is typhoid or not, where reliance is placed on the culture-plate appearance. But the plates were held in the ice box until after the first picking had been run through the necessary differential tests, so that the differentiation was more accurate than it otherwise would have been. The following records give the approximate number of typhoid colonies on the various plates for the different days of exposure.

Date	Days of Exposure	Character of Culture Plates
May 2	0	Typhoid type greatly predominating
May 3	1	Total colonies much diminished, about 8,000 typhoids per c.c.
May 4	2	About 700 typhoids on 1 plate; less on other 2 cultures
May 5	3	1 plate ($\frac{1}{10}$c.c.) had clump of about 50 typhoids, besides other scattering colonies. Both other plates contained a few
May 6	4	Several score typhoids on plate 1, 20–30 each on plates 2 and 3
May 8	6	40 typhoids on parchment sac from which 4 fished colonies were proven to be genuine typhoid. Only sporadic colonies on plates of other sacs
May 10	8	Character of plates completely changed. No typhoid observed except the single colony isolated from parchment sac
May 12	10	$\frac{1}{4}$ and $\frac{1}{2}$ c.c. water now used in culture plates. Germ content 300–500 colonies per plate, but all negative typhoid
May 15	13	1 c.c. cultures. Germ content trivial on D.-C. plates
May 19	17	1 c.c. cultures. D.-C. plates nearly sterile. Lactose agar about 150–200 colonies per c.c.

Thus, from an ocular inspection of the culture plates, as well as from the completed study of the isolated cultures, death is to be noted, in the course of a few days, of the hundreds of thousands of typhoid bacilli introduced into the lake water at the beginning. The great majority of these organisms disappeared in the course of a

few days (three or four), and after the lapse of six days, they could only be found in sporadic cases.

Series IV. Celloidin sac and glass tube filled with lake water, and immersed in lake water.—This series, run from August 15–24, 1905, included a celloidin sac and a glass tube of similar size. The dosage of the two containers was quite heavy, 2,275,000 and 1,706,000 typhoid bacilli per c.c. respectively. The temperature ranged from 21° to 23° C. during the work.

TABLE 9.

LONGEVITY OF B. TYPHOSUS IN CELLOIDIN SAC AND GLASS CONTAINER IN LAKE MENDOTA WATER.

	SAC 10 (CELLOIDIN)		SAC 11 (GLASS CONTAINER)		TOTALS	
	No. Col. Fished	No. Proven Typhoid	No. Col. Fished	No. Proven Typhoid	No Col. Fished	No. Proven Typhoid
1 hour	17	11	10	10	27	21
2 days	15	5	4	4	19	9
4 "	29	19	7	3	36	22
6 "	8	0	17	0	25	0
7 "	12	0	18	6	30	6
8 "	25	2	14	1	39	3
10 "	7	2	30	6	37	8
13 "	6	0	18	1	24	1
14 "	4	0	22	0	26	0
	123	39	140	31	263	70

In this series in the permeable sac, the inoculated typhoid was found as late as the 10th day, while in the glass container, immersed in the reservoir so as to maintain exactly the same temperature, it persisted until the 13th day. This result is in accord with data previously collected, where experiments have been carried on in glass receptacles, in which case it is generally found that the longevity of the typhoid organism is materially prolonged. For this reason, the earlier work on this question of longevity cannot be regarded as conforming to conditions that obtain in nature.

SYNOPSIS OF EXPERIMENTS WITH TYPHOID BACILLI EXPOSED TO NORMAL LAKE WATER.

In the foregoing series (I–IV) there is, on the whole, a striking agreement as to the length of time that the typhoid organism could be detected. In two respects considerable variation was to be observed in these series, viz., temperature of exposure and dosage, but the longevity of the introduced organism ranged through a comparatively narrow period of time (8–10 days). Two of these series were carried out in the winter, one in the spring, and another in the summer, when the average temperature of the water ranged

from 21° to 23° C. In Series III, which was made in May, the organism persisted for the minimum period of time, and it was thought possible that this might be ascribed to the higher temperature; but in Series IV, where the temperature was still higher, B. typhosus persisted for the usual period (8–10 days). In this case, though, the typhoid dosage was high, as the sacs were inoculated with approximately 2,000,000 organisms per c.c., a number much larger than usual.

In Table 10 is presented a summary of the results obtained in all these series, and, for purposes of comparison, the data collected last year by Professor Zeit in the work on Lake Michigan are also incorporated.

TABLE 10.

SUMMARY OF EXPERIMENTS ON LONGEVITY OF B. TYPHOSUS EXPOSED TO SURFACE WATERS (LAKE MENDOTA AND LAKE MICHIGAN).

LAKE MENDOTA.

SERIES	KIND OF SAC	SAC NO.	DAYS																	TOTAL NO. COL. FISHED	NO. PROVEN TYPHOID COL.
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
I	Agar	1	+		+	+	+	+	+	+	+	o	o	o	o	o				o	
	"	2	+		+	+	+	+	+	+	+	o	o			o				o	
	Celloidin	3	+		+	+	+														
	"	4	+		+	+	+	+	+	+	+	+1	+1		o					o	288	186
II	Agar	5				+			+		+				o						
	Celloidin	6				+			o		+1				o						54	11
III	Parchment	7	+	o	+	+	+		+		+1		o	o		o		o		o	
	"	8	+	+	+	o	+		+		o		o	o		o				o	
	Celloidin	9	+	+	+	+	o		+1		o		o	o		o		o			167	38
VI	Celloidin	10	+		+		+		o	o	+		+			o	o				
	Glass	11	+		+		+		o	+	+		+			+	o				213	63

LAKE MICHIGAN (ZEIT).

	Parchment	..	+	+	+		+		+	+	o		o	o							
	"	..	+	+	+	+		+			o	o									
	"	..	+	+	+	+		+		+	o		o		o						
	"	..	+	+	+	+	+	+	o	o	o	o				o					
	"	..	+	+	+		+	+	+	o	o	o									
	Celloidin	..	+	+	+	+	+	+	+	+	o	o	o	o	o	o					

+ means that typhoid was found more or less abundantly; o indicates complete disappearance of typhoid; +1, where figure "1" follows the plus sign, signifies that the positive typhoid findings were reduced to a single colony.

Comparing these two types of surface waters, one from an inland lake of moderate dimensions, the other from a very much larger water reservoir, it appears that the results of these two sets of experiments are not greatly different. In Professor Zeit's work, the average period of longevity was about seven days, while in our studies it has ranged from 8 to 10 days. Still, by far the most of the typhoid

organisms disappear before the end of a week. It is, however, necessary to set the limit at complete disappearance, although it has been generally noticed that there are often a few seemingly more resistant individual germs that persist for an appreciably longer time than the average.

PART II.

BACILLUS TYPHOSUS EXPOSED TO DIRECT INFLUENCE OF FRESH SEWAGE.

Having determined the relation of the typhoid organism to such natural surface waters as those of Lake Mendota, in which the normal bacterial content is relatively low, we next directed our attention to the question of the longevity of this organism when exposed to the influence of liquids rich in germ life and their products of growth. The previous work[1] on the waters of the Chicago Drainage Canal had indicated that the typhoid bacillus was unable to survive in highly polluted waters for as long a period of time as when exposed to a purer type of surface waters. This conclusion was made in a tentative way, but the importance of it in sanitary work is such that further study is desirable.

The purpose of the following series was to repeat this work on sewage in order to test the validity of the tentative conclusion previously drawn. For this purpose sacs were filled with fresh sewage, inoculated heavily with the same strain of the typhoid bacillus previously used, then immersed in a reservoir through which a stream of fresh sewage was allowed to flow.

Series V. Parchment, celloidin, and agar sacs filled with sewage and immersed in flowing fresh sewage.—Under the conditions of this series, the typhoid bacillus was exposed to the direct influence of the sewage organisms themselves, as well as their by-products of growth. In carrying out these experiments, it was necessary to use a much wider range of dilutions in making the cultures, in order to give the introduced bacillus most favorable opportunities for development. The typhoid dosage used was naturally much larger than that employed in the preceding cases. As was customary with the plate cultures made in the previous series, all plates were saved after they had been subjected to the usual examination, and all presumptive typhoid colonies marked and subcultured. The plates were then allowed to develop further, and after the discontinuance of sampling, the entire series, as a whole, was subjected to a comparative study, and the second crop of typhoid-like colonies removed. By subjecting the plate cultures to this comparative study, it is believed that it was possible to locate all typhoid organisms that developed on the plates.

In this series, three sacs (one each of celloidin, agar, and parchment), were filled with sewage and then immersed in flowing sewage. These sacs received respectively 1,500,000, 5,000,000, and 10,000,000 bacteria from a 24 hour culture of the "Y" strain.

Another celloidin sac, filled with sewage of the same composition, but not inoculated with typhoid, served as a control to study the course of the bacterial changes in the sewage itself.

[1] *Jour. Infec. Dis.*, 1904, 1, p. 641.

The sewage solution was made by mixing fresh human excreta (liquid as well as solid) with lake water and holding the mixture in a reservoir containing about 35 gallons. This reservoir was filled from time to time to maintain a continuous flow. As judged by appearance and odor, the sewage was fairly strong. Chlorine determinations were made at intervals, but naturally there was considerable fluctuation, depending upon the introduction of the urine. At the beginning of the experiment the sewage contained 244 parts of chlorine per million, while at the end there were 340 parts. The chlorine content was, however, lower than this during the progress of the experiment.

The following observations were made on the germ content of the sewage in the outside reservoir and within the control non-typhoid-infected sac.

TABLE 11.

BACTERIAL CONTENT PER C.C. OF SEWAGE.

	Days of Exposure	Outside Flowing Stream		Inside of Control Sac
		Lactose Agar	Drigalski-Conradi Medium	Lactose Agar
July 13	0	7,875,000	3,500,000	10,500,000
July 15	2		250,000	6,350,000
July 17	4		5,185,000	5,150,000
July 26	13		7,100,000	

In all cases an abundance of acid colonies on the D.-C. medium indicated the presence of sewage types. The temperature of the flowing liquid ranged from 21° to 29° C., with an average for the whole period of 24.8° C.

Results obtained in series V.—The examinations made on this series were begun on July 13 and continued on most of the sacs till July 27, covering a period of 14 days. At this time the character of the culture plates indicated that the typhoid type had entirely disappeared, and from previous experience it was deemed inadvisable to continue sampling longer. The results of this series are briefly summarized in Table 12, in which are given, for the respective days, (1) the total number of colonies picked, (2) the number which were regarded as presumptive typhoids on the basis of the litmus glucose agar test, and (3) the number of proven or verified typhoid colonies as determined by the agglutination and the differential culture tests.

The results obtained in all three sacs, including parchment, agar, and celloidin types, are in striking agreement with each other. In the three different kinds of permeable sacs employed, the results were identical. The last typhoid organism was found in each sac on the fifth day of exposure, but it is noteworthy that a marked decline

TABLE 12.

LONGEVITY OF B. TYPHOSUS EXPOSED IN PERMEABLE SACS FILLED WITH SEWAGE AND IMMERSED IN FLOWING SEWAGE.

DAYS	SAC 12 (AGAR)			SAC 13 (PARCHMENT)			SAC 14 (CELL.)			TOTALS DIFF. DAYS		
	No. Col. Fished	No. Pres. Typh. Found	No. Prov'n Ty-phoid	No. Col. Fished	No. Pres. Typh. Found	No. Prov'n Ty-phoid	No. Col. Fished	No. Pres. Typh.	No. Prov'n Ty-phoid	No. Col. Fished	No. Pres. Typh.	No Prov'n Ty-phoid
0.....	19	10	6	..	..	..	..	..	..	19	10	6
1.....	13	3	3	36	29	28	19	7	6	68	39	37
2.....	16	9	9	10	6	6	11	3	3	37	18	18
3.....	..	..	..	7	2	2	10	4	1	17	6	3
4.....	27	9	1	20	5	1	26	6	1	73	20	3
5.....	24	1	1	36	1	1	27	2	2	87	4	4
6.....	17	2	0	8	1	0	17	0	0	42	3	0
7.....	7	2	0	17	0	0	8	0	0	32	2	0
8.....	10	0	0	6	0	0	10	0	0	26	0	0
12.....	5	0	0	2	0	0	1	0	0	8	0	0
	138	36	20	142	44	38	129	22	13	409	102	71

set in considerably earlier. After 24 hours' exposure, of the 39 presumptive colonies removed from the D.-C. plates, 37 proved to be genuine typhoid. This percentage was maintained in equal ratio on the second day, but after this date fell rapidly, so that from the third to the fifth day of exposure, there could be found on cultures from each sac only one or two colonies that proved to be B. typhosus. After this date, 108 more colonies were taken off, but nearly all were eliminated by the litmus glucose test, and all proved negative typhoid on the application of the agglutination test.

TABLE 13.

LONGEVITY OF B. TYPHOSUS EXPOSED TO THE DIRECT ACTION OF SEWAGE BACTERIA.

DAYS	SAC 15 (PARCHMENT)			SAC 16 (CELLOIDIN)			TOTALS		
	No. Col. Col. Fished	No. Pres. Typh.	No. Proven Typh.	No. Col. Fished	No. Pres. Typh.	No. Proven Typh.	No. Col. Fished	No. Pres. Typh.	No. Proven Typh.
0.....	16	16	16	..	..	..	16	16	16
1.....	3	3	3	50	7	7	53	10	10
2.....	38	15	15	26	3	3	64	18	18
3.....	22	10	10	20	2	2	42	12	12
4.....	15	0	0	8	0	0	23	0	0
5.....	7	0	0	13	0	3	20	0	3
6.....	7	0	0	6	0	0	13	0	0
8.....	3	0	0	2	0	0	5	0	0
10.....	3	0	0	5	0	0	8	0	0
14.....	3	0	0	4	0	0	7	0	0
16.....	1	0	0	7	0	0	8	0	0
	118	44	44	141	12	15	259	56	59

Series VI. Parchment and celloidin sacs filled with sewage and immersed in sewage.—The same method of arrangement, as detailed in preceding series, was followed in this series, which was begun on August 15, 1905, and continued until

August 29. A parchment and a celloidin sac, heavily seeded with typhoid organisms (4,350,000 and 5,800,000 bacteria, respectively), were filled with fresh sewage and immersed as before in flowing sewage. Bacteriological examinations made with D.-C. media on the sewage showed usually a germ content ranging from 1,250,000–6,600,000. The chlorine content varied from 150 to 200 parts per million. The range in temperature was from 22° to 25° C. The results obtained are shown in Table 13.

In this series the disappearance of the typhoid organism was even more rapid than in the foregoing test. Two hundred and fifty-nine cultures were removed, and of these 59 proved to be typhoid. In the parchment sac none were found after the third day, while in the celloidin sac the presumptive typhoids were sparse after two or three days, and had entirely disappeared after five days.

These results are in harmony with those obtained the year before on the Chicago Drainage Canal, in that in both sets of experiments, the longevity of the typhoid organism was much shorter when exposed in sewage than in lake water. In the earlier series of studies, the introduced organism was not found after the third day, while practically the same result was obtained in both of the series here recorded, although scattering colonies were found on the culture plates as late as the fifth day.

Taking into consideration, the results obtained in both the Chicago series and those here described it would seem that the data obtained warrant the conviction that the typhoid organism is unable to retain its vitality as long when immersed in sewage as it does when in contact with lake water. This fact being determined, the next question of interest is to find the cause of this phenomenon. Is this diminished longevity due (1) to the direct action of the sewage bacteria themselves, or (2) to the by-products which this type of life produces? By means of the technical methods here used, it was thought that this problem might be solved by exposing the typhoid organisms in sacs filled with lake water to the influence of flowing sewage. If the period of longevity of the inoculated organism was fully as great when the lake water sacs were immersed in sewage as when subjected to the current of flowing lake water, then it would seem that the influence of the soluble growth products of sewage bacteria would be of no effect. On the other hand, if they died as quickly as they did in sewage, or nearly as soon, then it would appear that the soluble substances pass-

ing from the sewage outside through the permeable membranes exerted a harmful action.

To test this hypothesis, several series were instituted in which the inoculated organism was exposed in the way just mentioned.

PART III.

BACILLUS TYPHOSUS EXPOSED TO THE INFLUENCE OF DIFFUSIBLE SEWAGE BY-PRODUCTS AND TO WATER BACTERIA.

Three different sets of experiments were made on this point, in which sacs were filled with raw lake water, then inoculated with B. typhosus and the whole immersed in flowing sewage. Series VII, consisting of two sacs each of celloidin, parchment, and agar, was inoculated with 190,000–300,000 organisms per c.c. and run from May 2 to May 19 of this year.

Two other series (VIII and IX), each consisting of a celloidin and a parchment sac, were inoculated, one with a heavy seeding, the other with a light seeding, of B. typhosus, and immersed in flowing sewage.

Series VII. Celloidin, parchment, and agar sacs, filled with lake water and immersed in flowing, fresh sewage.—Six sacs were employed in this series, two each of the three types. The sewage on the outside of the sacs was fairly strong, as judged by sight and smell. It was quite turbid, and of a dark brown color, due to accumulation of organic matter, which collected on the walls of the containing reservoir, especially at and near the surface. A cover-glass preparation showed a matrix of colorless filaments, brownish cells, and amorphous matter. From time to time the germ content and the chlorine content of this flowing liquid was ascertained to gauge its relative condition. The bacterial counts were made after 24 hours' incubation. A longer period of incubation would doubtless have increased materially the figures given.

TABLE 14.

CHARACTER OF SEWAGE AS TO CHLORINE AND BACTERIAL CONTENT.

	Chlorine Pts. per 1,000,000	Bacteria per c.c. Lactose Agar	Litmus Lactose Agar
May 2	...	128,250	Numerous acid and gas colonies
May 4	175	.....	
May 5	...	80,000	Gas formers and acid colonies abundant
May 6	125	.....	
May 8	22	61,500	Sewage forms abundant
May 10	...	160,000	Acid and gas colonies numerous
May 11	15	.....	
May 12	24	.....	
May 13	27	.....	
May 15	24	.....	
May 17	20	.....	
May 18	70	.....	

The variation in chlorine content was doubtless occasioned by the somewhat irregular addition of the urine to the sewage. The temperatures ranged in this series from 15° to 19° C., with an average temperature of 16.7° C. All three kinds of sacs were used in this series,

two sacs of each being employed. At the outset an attempt was made to determine the quantitative condition of the respective sacs in a manner similar to that indicated in Series I, but this was discontinued during the progress of the experiment when it became evident that a large proportion of the organisms found on the culture plates were not typhoid, but were water saprophytes. The germ content of the different sacs at the beginning ranged from 190,000 to 330,000 per c.c., which was, of course, mainly B. typhosus, as the water used in the sacs was lake water, containing not more than 150–300 bacteria per c.c. On the second and third days of exposure the total germ content in the different sacs had fallen 50–90 per cent, but after this date a marked increase occurred, reaching in the course of a few days several hundred thousand bacteria per c.c. It would appear from these data that the water bacteria originally introduced, with the lake water, into the sac, had multiplied extensively. This is a matter of import, as this multiplication might exert some effect upon the longevity of the pathogenic organism with which they were exposed.

A more important fact, however, is that this demonstrates the permeability of the sacs to substances from without, for this enormous development far transcends any growth that would have occurred without influx of organic matter from the outside. That none of this growth was due to the admission of the sewagic forms from the outside is conclusively shown by the entire absence of any gas forming acid colonies on all the plates for about two weeks. The colonies growing on the Drigalski-Conradi medium were uniformly of some shade of blue or grayish color. Not until the 13th day did any acid colony appear. On this day a single acid colony developed in cultures made from one of the parchment, and also from one of the agar, sacs. This condition was also observed the next day in the same parchment sac, and in the other agar sac. These results, therefore, speak strongly for the integrity of the sacs for a sufficiently long period, as they were surrounded continually with water containing hundreds, and often thousands, of acid and gas generating forms per c.c.

The records of the plates as to their colony appearance is herewith presented:

Date	Days of Exposure	Character of Culture Plates
May 2	0	All sacs cultured immediately after installation and found to contain very nearly a pure typhoid culture
May 3	1	Major portion of colonies typhoid on parchment (Sac 18); agar sac (Sac 22) very nearly a pure culture of typhoid
May 4	2	Presumptive typhoids apparently few on agar (Sac 21). Suspected colonies picked failed to grow
May 5	3	About one-half on celloidin (Sac 19) appear to be typhoid. Typhoid abundant on agar (Sacs 21 and 22)
May 7	5	Typhoid abundant on celloidin (Sac 19)
May 8	6	Typhoid quite numerous on celloidin (Sac 20)
May 9	7	Culture badly mixed. Suspected typhoids very sparingly present
May 10	8	Colonies on plates from agar sac appear negative typhoid
May 11	9	Several colonies on celloidin (Sac 19) resemble typhoid, but only one proves positive
May 13	11	All colonies evidently negative typhoid. Total colony count falling rapidly

The results obtained from the testing of the cultures fished from the culture plates are expressed in Table 15.

TABLE 15.

LONGEVITY OF B. TYPHOSUS IN PERMEABLE SACS FILLED WITH LAKE WATER AND EXPOSED TO INFLUENCE OF FRESH SEWAGE.

	Sac 17 Parch.		Sac 18 Parch.		Sac 19 Cell.		Sac 20 Cell.		Sac 21 Agar		Sac 22 Agar		Totals	
	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Typh. Col. Found	No. Col. Fished	No. Proven Typh.
1 hour	6	6	4	3	3	2	3	3	5	5	4	4	25	23
1 day	3	1	7	7	3	3	3	3	5	3	4	4	25	21
2 days	4	1	..	..	5	2	7	2	4	0	5	0	25	5
3 "	4	0	8	4	5	4	2	2	4	1	3	2	26	13
4 "	6	2	..	..	4	2	7	4	1	1	6	1	24	10
5 "	6	1	7	0	10	7	..	..	15	2	..	..	38	10
6 "	..	..	..	..	..	..	7	5	..	..	6	2	13	7
7 "	10	2	3	0	3	0	..	..	5	0	..	..	21	2
8 "	..	..	6	0	..	..	7	2	..	..	5	1	18	3
9 "	7	0	5	0	9	1	..	..	4	0	6	..	25	1
10 "	..	..	1	0	..	..	4	0	..	0	5	0	10	0
11 "	5	0	..	..	5	0	4	0	4	0	6	1	24	1
13 "	4	0	..	..	5	0	5	0	3	..	3	0	20	0
15 "	2	0	1	0	1	0	1	0	1	..	1	0	7	0
17 "	..	..	..	..	4	0	5	0	..	..	..	..	9	0
	57	13	42	14	57	21	55	21	51	12	48	15	310	96

In the parchment series, the character of the colonies in the culture plates had changed by the seventh day so that typhoid-like organisms were very sparingly present. The results in all of the sacs, as a whole were, however, more divergent than in any of the preceding series. An average of the whole six sacs used was something over seven days, not greatly different from those made in flow-

ing lake water. Even though the divergence in results was considerable, the sacs in this series showed a greater degree of longevity than in those in which the typhoid organisms were immersed in sewage. This would seem to indicate that the sewage in the outer reservoir exerted little or no effect on the vitality of the typhoid within. With such divergent results it is, however, impossible to draw any conclusion, and the further data on this point, presented in Series VIII and IX, are necessary as a basis for deductions.

Series VIII. Celloidin and parchment sacs filled with lake water and immersed in flowing sewage.—This series consisted of three sacs: one celloidin and one parchment, filled with raw lake water and inoculated with the usual typhoid strain; also, a third celloidin sac filled with lake water, but uninfected with the pathogenic organism. These three sacs were immersed in a bath of flowing sewage. The exposure was continued for a period of 14 days. The temperature ranged from 21° to 29° C., with an average of about 25° C. The sewage in the outside receptacle was fresh and quite strong. Chlorine determinations showed a range from 241 to 340 parts per million. The bacterial content of this outside sewage was usually from 3,500,000 to 7,000,000 organisms per c.c.

A bacterial determination on Drigalski-Conradi medium was made of the uninfected celloidin sac, and it is interesting to note the enormous development that occurred in the lake water submerged in the sewage, as shown in the following data.

TABLE 16.

BACTERIAL CONTENT PER C.C. OF LAKE WATER IN CELLOIDIN SAC IMMERSED IN FLOWING SEWAGE.

	Period of Immersion (Days)	Bacteria per c.c.	Acid Colonies
June 13	0	30	0
June 15	2	10,500	0
June 18	5	10,950,000	0
June 25	12	26,600,000	0

The above data are of importance, as showing the course of the changes that occur in the permeable sacs when immersed in a medium containing a large amount of organic matter. This sac filled with water showed a degree of growth that is almost unparalleled, multiplying in the course of 12 days nearly a million-fold. The fact that no acid colonies developed on these plates made from the water is proof of the integrity of the sacs. This bacterial growth is far in excess of that which occurs in the permeable sacs when immersed in flowing lake water, and would seem to be explained on the assumption that nutrient substances of a diffusible character are capable of passing through the membrane, from the sewage outside.

The record of the longevity of the typhoid type is shown below.

TABLE 17.

LONGEVITY OF B. TYPHOSUS IN CELLOIDIN AND PARCHMENT SACS FILLED WITH LAKE WATER AND IMMERSED IN FLOWING SEWAGE.

	SAC 23 (PARCHMENT)		SAC 24 (CELLOIDIN)		
	No. Colonies Fished	No. Proven Typhoid	No. Colonies Fished	No. Presumptive Typhoid	No. Proven Typhoid
0 days	..	..	13	12	10
1 day	9	0			
2 days	6	0	9	0	0
4 "	13	5	10	8	8
6 "	18	9			
10 "	21	4	Leak discovered, further sampling discontinued		
11 "	22	0			
12 "	17	0			
13 "	15	0			
14 "	16	0			

In this series an accident happened to the celloidin sac. In some way a leak developed, which fact could of course be quickly detected by the appearance of red colonies on the D.-C. plates, whereas the lake water, to begin with, was free from all acid-producing forms. From Sac 23 it happened that no cultures were made between the 6th and the 10th days, so that the history at this point is not as complete as it should have been, but it is significant that the typhoid organisms were readily recovered on the 10th day. A large number of cultures were taken after this date (70 on four successive days), but in no case was any organism found that even simulated the typhoid type of colony or proved positive upon the application of the differential tests.

When these results are compared with those contained in Series VII, it appears in both series that the inoculated typhoid persisted for a considerably longer period than in case of direct contact with sewage itself.

A rapid change, as usual, took place in the general character of the culture plates. On July 14, the second day after the lake water was infected, the major portion of the plate cultures showed typical typhoid colonies. In the course of a few days the total germ content per c.c. of this sac increased rapidly, due to the growth of the water organisms. On the 17th, five days after starting the experiment, about one-third (16,000 organisms) of the total colony count was still B. typhosus. By the 19th this number had fallen to about 8,000 per c.c. On the 23d both D.-C. plates contained a few presumptive typhoids, but the later plates showed totally aberrant forms.

Series IX. Celloidin and parchment sacs filled with lake water and immersed in flowing sewage.—This series was started on August 15 and continued till the 29th. Two sacs, one of celloidin and the other of parchment, were inoculated lightly with

100,000 and 200,000 typhoid bacilli, respectively. The findings in this test are expressed in Table 18.

TABLE 18.

LONGEVITY OF B. TYPHOSUS IN SACS FILLED WITH LAKE WATER AND EXPOSED TO THE ACTION OF FLOWING SEWAGE.

DAYS	SAC 25 (CELLOIDIN)			SAC 26 (PARCHMENT)			TOTALS		
	No. Col. Fished	No. Pres. Typh.	No. Proven Typh.	No. Col. Fished	No. Pres. Typh.	No. Proven Typh.	No. Col. Fished	No. Pres. Typh.	No. Proven Typh.
0.....	12	12	12	14	14	13	26	26	25
2.....	12	2	2	12	1	1	24	3	3
4.....	17	0	0	21	1	1	38	1	1
6.....	26	11	11	30	11	11	56	22	22
7.....	10	6	6	59	14	14	69	20	20
8.....	21	0	0	31	1	1	52	1	1
10.....	3	0	0	5	0	0	8	0	0
13.....	7	0	0	9	0	0	16	0	0
14.....	7	0	0	12	0	0	19	0	0
	115	31	31	193	32	41	308	53	72

The most evident change in this series came suddenly on the eighth day. In the celloidin sac no typhoids were found on this date, or at any subsequent time, and in the parchment sac only one typhoid colony appeared on the eighth day, and none thereafter.

The results of these three foregoing series (see Summary, Table 19) show some variation in the longevity of the typhoid organism, but not more than was observed in the earlier series, where the exposure was in lake water alone. In general the typhoid bacillus persisted in these cases for about seven or eight days, with an occasional instance where vitality was prolonged for 10 or 11 days. These results stand in striking contrast to those obtained in the sewage series. This variation in longevity is brought out under optimum conditions in the case of two of the series that were run under conditions identical except as to the nature of the liquid in the sac.

Series VI and IX were immersed in the same sewage and run at the same time. Series VI contained sacs filled with lake water, while in Series IX the sacs were filled with sewage. The sewage series also received much the heavier seeding. As to results, the typhoid organism died in the sewage sacs in from three to five days, while in the sac filled with lake water, but immersed in same stream of sewage, it lived seven to eight days.

To permit of more ready comparison of results, reference can be made to Table 19, in which is summarized the results of all of the

experiments made on the sewage series and those in which sacs were filled with lake water and immersed in sewage.

TABLE 10.

SUMMARY OF EXPERIMENTS WHERE EXPOSURE WAS MADE IN SEWAGE SACS, ALSO IN LAKE WATER SACS IMMERSED IN FLOWING SEWAGE.

	Series	Sac No.	Kind of Sac	Days 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total No. Col. Fished	No. Prov'n Typhoid Col.
Sewage sacs	V	12	Agar.....	+	+	+		+1	+1	o	o	o				o				
		13	Parchment.		+	+	+	+1	+1	o	o	o				o				
		14	Celloidin..		+	+	+1	+1	+	o	o	o				o			409	71
	VI	15	Parchment.	+	+	+	+	o	o	o		o		o			o	o		
		16	Celloidin..		+	+	+	o	+	o		o		o			o	o	259	59
Lake water sacs	VII	17	Parchment.	+	+	+	o	+	+		+		o		o		o	o		
		18	Parchment.	+	+		+		o		o	o	o	o				o		
		19	Celloidin..	+	+	+	+	+	+		o		+1		o		o	o		
		20	Celloidin..	+	+	+	+	+		+		+		o	o		o	o		
		21	Agar.....	+	+	+	+	+	+		o		o		o		o	o		
		22	Agar	+	+	o	+	+		+		+		o	+1		o	o	310	96
	VIII	23	Parchment.		o	o		+		+				+	o	o	o	o		
		24	Celloidin..	+		o		+		lea	k d	isc	ove	red					169	36
	IX	25	Celloidin..	+		+		o		+	+	o		o			o	o		
		26	Parchment.	+		+		+		+	+	+1					o	o	308	72
																			1,455	334

The evident conclusion which these data support is that the results obtained in the three series (VII–IX) in which the sacs were filled with typhoid-infected lake water and immersed in sewage are much more nearly in accord with the series exposed to running water (I–IV) than to those subjected to the direct action of sewage (V–VI). This would seem to indicate that the more destructive influence of sewage was not exerted unless the typhoid organism was in intimate contact with the sewage bacteria themselves.

Series X. One more possible combination existed, which was tried in order to test all aspects of the question at issue. Sacs filled with water had been immersed in flowing water; those filled with sewage had been placed in a sewage bath; and others filled with water had also been subjected to the influence of flowing sewage. The remaining combination of sewage-filled sac immersed in flowing water was therefore tried. In this case only a single sac was used (parchment). It was heavily inoculated with typhoid, 4,350,000 organisms per c.c. of a 24 hour culture.

A rapid death of the introduced germ occurred in this sac in the course of a few days. The latest recovery was on the fifth day, although even prior to this it had been greatly reduced in numbers. This result is practically the same as the sewage sacs immersed in

TABLE 20.

LONGEVITY OF B. TYPHOSUS IN PARCHMENT SAC FILLED WITH SEWAGE AND IMMERSED IN WATER.

DAYS	SAC 27 (PARCHMENT)		
	No. Colonies Fished	No. Pres. Typhoid	No. Proven Typhoid
0	20	19	19
1	2	2	2
2	30	6	6
3	18	2	2
4	12	0	0
5	12	1	1
6	4	0	0
8	0	0	0
10	5	0	0
13	17	0	0
14	9	0	0
	129	30	30

sewage. Although undoubtedly considerable diffusion of soluble substances would occur in this case, no material variation in the longevity of the pathogenic organism was observed. This is in accord with all of the previous results, and indicates that the longevity of B. typhosus is diminished when the organism is in direct contact with sewage bacteria.

In Table 21 are compiled the results of all experiments, expressed in days. This signifies the maximum period of time in each experiment during which the typhoid bacillus could be recovered. For findings other than the end results, comparative summaries may be found in Tables 10 and 19.

TABLE 21.

SUMMARY OF RESULTS.

B. TYPHOSUS EXPOSED TO	SACS IMMERSED IN	SERIES NO.	LONGEVITY OF TYPHOID (IN DAYS)			
			Agar	Celloidin	Parchm't	Glass
Lake water	Lake Water	1	8, 8	8, 10		
		2	8	8		
		3		6	6, 8	
		4		10		13
Sewage	Sewage	5	5	5	5	
		6		5	3	
Lake water	Sewage	7	5	8, 9	3, 7	
		8		?	10	
		9		7	8	
Sewage	Lake water	10			5	

CONCLUSIONS.

1. Three types of permeable sacs (celloidin, parchment, and agar films) were employed to hold the typhoid organism imprisoned while it was exposed to the influence of water and sewage bacteria.

2. Tests made with chlorides, sugar, and peptone indicate that these sacs were readily permeable, while other tests demonstrated that they were wholly germ-tight.

3. In four series of examinations where B. typhosus was exposed to the action of flowing lake water (Mendota), the longevity of the organism ranged from 8 to 10 days, agreeing quite closely with the experiments previously reported on Lake Michigan water under similar experimental conditions.

4. Where B. typhosus was exposed directly to the action of sewage bacteria, its longevity was greatly diminished, three to five days being the longest time for which the organism could be recovered.

5. When the typhoid organism was exposed to the diffusible products of sewage bacteria, and not to the direct action of the organisms themselves, as was the case when the typhoid infected lake water-filled-sacs were exposed to flowing sewage, the longevity of the inoculated pathogenic form was increased. The results, although more variable than in either of the preceding cases, agreed more closely with those obtained in the lake-water series than with those exposed to the action of sewage itself. This would seem to indicate that the direct contact with sewage organisms was more detrimental to the vitality of the typhoid organism than the diffusible by-products of sewage forms.

6. Where typhoid-infected sewage-filled sacs were exposed to water, the longevity of B. typhosus was the same (five days) as where the bathing liquid was sewage. This again seems to indicate that the question of longevity is more dependent upon the actual contact with sewage types than it is upon contact with by-products capable of diffusion through these permeable membranes.

7. The uniformity noted in the results obtained in this investigation, and their confirmation of the work of the preceding year on the waters of Lake Michigan and the Chicago Drainage Canal would now seem to warrant the definite conclusion that the longevity of the typhoid bacillus in waters is materially affected by the germ content of its surroundings. In waters highly polluted with saprophytic bacteria, such as is the case in sewage, this disease organism is unable to survive for more than a few days (three to five in the experiments here described), a period of time materially shorter than that which is noted in normally unpolluted waters.